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Purification and Partial Characterization of the Major Outer Membrane Protein of *Chlamydia trachomatis*

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Elementary bodies (EB) of *Chlamydia trachomatis* serotypes C, E, and L2 were extrinsically radioiodinated, and whole-cell lysates of these serotypes were compared by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Autoradiography of the polypeptide profiles identified a major surface protein with an apparent subunit molecular weight of 39,500 that was common to each *C. trachomatis* serotype. The abilities of nonionic (Triton X-100), dipolar ionic (Zwittergent TM-314), mild (sodium deoxycholate and sodium *N*-lauroyl sarcosine), and strongly anionic (SDS) detergents to extract this protein from intact EB of the L2 serotype were investigated by SDS-PAGE analysis of the soluble and insoluble fractions obtained after each detergent treatment. Only SDS readily extracted this protein from intact EB. Sarkosyl treatment selectively solubilized the majority of other EB proteins, leaving the 39,500-dalton protein associated with the Sarkosyl-insoluble fraction. Ultrastructural studies of the Sarkosyl-insoluble EB pellet showed it to consist of empty EB particles possessing an apparently intact outer membrane. No structural evidence for a peptidoglycan-like cell wall was found. Morphologically these chlamydial outer membrane complexes (COMC) resembled intact chlamydial EB outer membranes. The 39,500-dalton outer membrane protein was quantitatively extracted from COMC by treating them with 2% SDS at 60°C. This protein accounted for 61% of the total COMC-associated protein, and its extraction resulted in a concomitant loss of the COMC membrane structure and morphology. The soluble extract obtained from SDS-treated COMC was adsorbed to a hydroxylapatite column and eluted with a linear sodium phosphate gradient. The 39,500-dalton protein was eluted from the column as a single peak at a phosphate concentration of approximately 0.3 M. The eluted protein was nearly homogeneous by SDS-PAGE and appeared free of contaminating carbohydrate, glycolipid, and nucleic acid. Hyperimmune mouse antiserum prepared against the 39,500-dalton protein from serotype L2 reacted with *C. trachomatis* serotypes Ba, E, D, K, L1, L2, and L3 by indirect immunofluorescence with EB but failed to react with serotypes A, B, C, F, G, H, I, and J, with the *C. trachomatis* mouse pneumonitis strain, or with the *C. psittaci* feline pneumonitis, guinea pig inclusion conjunctivitis, or 6BC strains. Thus, the 39,500-dalton major outer membrane protein is a serogroup antigen of *C. trachomatis* organisms.

Chlamydia are obligate intracellular bacteria characterized by a unique and complex growth cycle. Phylogenetically, the chlamydiae are closely related to gram-negative organisms. In particular, both gram-negative bacteria and chlamydiae have outer membranes that respond similarly to treatment with ethylenediaminetetraacetic acid (EDTA) under alkaline conditions (33) or exposure to polymyxin B (26). These findings suggest both biochemical and structural similarities between chlamydial and gram-negative bacterial outer membranes. The outer membrane of gram-negative bacteria has been intensely investigated with respect to its composition and function (4). Unique proteins, termed "matrix" or "major" proteins, may account for as much as 60% of the total outer membrane protein (22). The function of these proteins with respect to structure and antigenic character is currently a topic of intense study. For certain members of the *Enterobacteriaceae*, the matrix proteins are bound tightly but not

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covalently to the peptidoglycan (35). These proteins form hydrophobic diffusion pores (31, 32) that span the bacterial outer membrane. Similar proteins are found in the outer membrane of the genus *Neisseria* (7, 14) and, because of their abundance and location, investigators have been interested in their immunobiological functions. The major or principle outer membrane protein of *Neisseria gonorrhoeae* organisms is believed to be an important serotyping antigen (13, 15) and may play a role in gonococcal virulence (11). Little such information is available concerning the native chlamydial outer membrane. Because some properties of chlamydial and gram-negative bacterial outer membranes appear similar, we asked whether chlamydiae have major outer membrane proteins with properties and functions that parallel those of gram-negative bacteria.

In this study, we found that *C. trachomatis* organisms possess a major outer membrane protein that resembles the matrix proteins of gram-negative bacteria. Procedures are described for the isolation and purification of this protein, and evidence is presented suggesting its function as a structural protein that contributes to the rigidity of the chlamydial elementary body (EB). Furthermore, preliminary antigenic characterization of the major outer membrane protein indicates that it is a serogroup antigen of *C. trachomatis* organisms.

MATERIALS AND METHODS

Organisms and growth conditions. The following *C. trachomatis* strains were used: L2/434/Bu(L2), E/UW-5/Cx(E), and C/TW-3/OT(C). Chlamydiae were grown in HeLa 229 cells as described previously (3). The L2 strain was also grown in suspension cultures of L-929 cells (3). L-cell-propagated L2 organisms were used for the isolation and purification of the 39,500-dalton protein.

Purification of chlamydiae. Chlamydiae were harvested from HeLa cell monolayers grown in 150-cm^2 polystyrene culture flasks (Corning Glass Works, Corning, N.Y.), with ≥90% of the cells containing inclusions at 48 h postinoculation. Medium was poured off, and cells were removed with 4-mm glass beads and 10 mm of cold Hanks balanced salt solution. The cell suspensions were pooled, and the cells were ruptured by sonication (Braunsonic model 1510). This suspension was centrifuged at 500 × *g* for 15 min at 4°C. The supernatants were layered over 8 ml of a 35% (vol/vol) Renografin solution (diatrizoate meglumine and diatrizate sodium, 76% for injection; E. R. Squibb & Sons, Princeton, N.J.) in 0.01 M HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) containing 0.15 M NaCl and then centrifuged at 43,000 × *g* for 1 h at 4°C in an SW27 rotor (Beckman Instruments, Inc., Fullerton, Calif.). The pellets were suspended in 0.01 M sodium phosphate (pH 7.2) containing 0.25 M sucrose and 5 mM L-glutamic acid (SPG), pooled, and layered over discontinuous Renografin gradients (13 ml of 40%, 8 ml of 44%, and 5 ml of 52% Renografin, vol/vol). These gradients were centrifuged at 43,000 × *g* for 1 h at 4°C in an SW27 rotor. The EB bands, located at the 44/52% Renografin interface, were collected, diluted with 3 volumes of SPG, and then centrifuged at 30,000 × *g* for 30 min. The EB pellets were washed in SPG to remove residual Renografin. Purified EB were suspended in SPG and stored at −80°C. The purity of EB preparations was determined by electron microscopy and Macchiavello-stained smears.

Electron microscopy. For electron microscopy, samples were fixed in 1.5% glutaraldehyde in 0.1 M sodium cacodylate hydrochloride buffer (pH 7.4) containing 1% sucrose, postfixed in 1% OsO_4 in acetate-Veronal buffer (pH 7.6) containing 5% sucrose, stained en bloc in 0.5% uranyl acetate in the same buffer containing 4% sucrose, dehydrated in a series of alcohols and propylene oxide, and embedded in Epon 812. Sections were stained with uranyl acetate and lead citrate and were examined with a Siemens Elmiskop IA electron microscope.

Chemical procedures. Protein was estimated by the method of Lowry et al. (21), using bovine serum albumin (Sigma Chemical Co., St. Louis, Mo.) as a standard. Nucleic acid was estimated spectrophotometrically by optical density at 280/260 nm ($OD_{280/260}$) according to Layne (20). Inorganic phosphorus was assayed by the Sigma-modified Fiske and Subbarow assay (Sigma Technical Bulletin no. 670).

Purified protein preparations were analyzed for the presence of 2-keto-3-deoxyoctonate by the method of Weissback and Hurwitz (41) as modified by Osborn (34), using 2-keto-3-deoxyoctonate (Sigma) as a standard. The absorbance was measured at 548 nm. The presence of glycolipid and nucleic acid was determined by staining polyacrylamide gels with Stains-All (Bio-Rad Laboratories, Richmond, Calif.) as described by King and Morrison (16). Carbohydrate was determined by staining gels by the periodic acid-Schiff procedure of Fairbanks et al. (5).

SDS-PAGE. Chlamydial proteins were electrophoresed on 12.5% acrylamide slab gels in the discontinuous tris(hydroxymethyl)aminomethane (Tris)-glycine system described by Laemmli (19). The ratio of acrylamide to *N,N'*-methylenebisacrylamide was 30:0.8 in both the 12.5% separating gel and the 5% stacking gel. Before electrophoresis, samples were mixed with an equal volume of solubilizing solution (0.1 M Tris-hydrochloride, pH 6.8) containing 2.5% sodium dodecyl sulfate (SDS; BDH, Poole, England), 5% 2-mercaptoethanol, 20% glycerol, and 0.001% bromophenol blue and boiled for 10 min. Polyacrylamide gel electrophoresis (PAGE) in Tris-glycine buffer (pH 8.6) containing 0.1% SDS was carried out at a constant current of 25 mA. Gels were stained in 0.25% Coomassie brilliant blue R-250 in 7% acetic acid and 30% methanol. The protein standards used for estimating chlamydial protein molecular weights were: phosphorylase b (94,000), bovine serum albumin (67,000), ovalbumin (43,000), carbonic anhydrase (30,000), soybean trypsin inhibitor (20,100), and α-lactalbumin (14,400) (Pharmacia Fine Chemicals, Inc., Piscataway, N.J.).

Extrinsic ^{125}I iodination of chlamydial EB. Purified EB were radiolabeled according to the method of Marchalonis (24) with some modification. The reac-

tion mixture consisted of the following: 200 μl of purified EB (150 to 200 μg of protein) in SPG containing 5 μM KI; 5 μl of carrier-free $Na^{125}I$ (500 μCi) (New England Nuclear Corp., Boston, Mass.), and 10 μl of lactoperoxidase (Sigma) (2 mg/ml in distilled water). The reaction was initiated by the addition of 5 μl of 0.015% H_2O_2. Three additional 5-μl portions of H_2O_2 were then added at 5-min intervals. Next, 3 ml of SPG was added, and this suspension was layered over 1 ml of 30% Renografin and centrifuged at 43,000 × g for 1 h at 4°C. The pellet was suspended in 200 μl of SDS-solubilizing solution, boiled for 10 min, and centrifuged at 30,000 × g for 30 min. The supernatant (chlamydial whole-cell lysate) was retained for SDS-PAGE. After SDS-PAGE of chlamydial proteins, gels were stained with Coomassie blue, destained, and dried under vacuum on Whatman 3MM chromatography paper. Kodak X-Omat R film (Eastman Kodak Co., Rochester, N.Y.) was used for autoradiography of dried gels.

Effect of different detergents on the extraction of the 39,500-dalton protein. Purified L2 EB (18 mg of protein) were suspended in 6 ml of 0.01 M sodium phosphate, pH 8.0, containing 0.15 M NaCl (PBS). This suspension was divided equally into 1-ml portions and centrifuged at 30,000 × g for 15 min. Each of the six EB pellets was then suspended in 1 ml of PBS containing 1.5 mM EDTA and one of the following detergents: (i) 2% SDS; (ii) 2% sodium *N*-lauroyl sarcosine (Sarkosyl; Sigma); (iii) 2% Zwittergent TM-314 (CalBiochem, San Diego, Calif.); (iv) 2% sodium deoxycholate (Sigma); or (v) 2% Triton X-100 (Sigma). A single EB pellet was suspended in PBS containing 1.5 mM EDTA only as a control for detergent solubilization. These suspensions were sonicated briefly to disrupt clumped EB, incubated for 1 h at 37°C, and then centrifuged at 100,000 × g for 1 h at 37°C. The soluble supernatants were collected, and the extracted proteins were analyzed by SDS-PAGE. The insoluble pellets were each washed once in PBS and then suspended in 1 ml of the SDS buffer solution. These suspensions were incubated at 37°C for 1 h and centrifuged as before, and the supernatants were analyzed by SDS-PAGE.

Isolation of chlamydial outer membrane complexes (COMC) by Sarkosyl extraction of intact EB. *C. trachomatis* L2 EB were suspended (approximately 5 mg of EB protein per ml) in 5 ml of PBS, pH 8.0, containing 2% Sarkosyl and 1.5 mM EDTA. This suspension was incubated at 37°C for 1 h and then centrifuged at 100,000 × g for 1 h. The insoluble pellet was resuspended in the same Sarkosyl buffer and centrifuged as before. The pellet was washed twice in PBS to remove excess detergent and then suspended in 0.02 M sodium phosphate, pH 8.0, containing 10 mM $MgCl_2$ and 25 μg of deoxyribonuclease I (Worthington Biochemicals Corp., Freehold, N.J.) and ribonuclease (Millipore Corp., Bedford, Mass.). This suspension was incubated for 2 h at 37°C and centrifuged, and the insoluble pellet was washed twice with PBS to remove any remaining nucleases. This Sarkosyl-insoluble material consisted of COMC.

Effect of temperature on SDS extraction of the 39,500-dalton protein from isolated COMC. A suspension of L2 COMC, prepared from 18 mg of EB protein, was divided into 5 equal volumes and centrifuged at 100,000 × g for 1 h. The pelleted COMC were then suspended in 1 ml of 2% SDS buffer, and each suspension was incubated at 37, 45, 60, or 80°C for 1 h or at 100°C for 10 min. These suspensions were then centrifuged at 100,000 × g for 1 h at 37°C, and the soluble supernatant fractions were saved. Each of the insoluble pellets was then suspended in 1 ml of SDS buffer, incubated at 100°C for 10 min, and centrifuged as before. Equal volumes of these soluble supernatants and those obtained from the preceding SDS extraction step were analyzed by SDS-PAGE. Densitometric scans of the stained gel electropherograms were done to determine the quantity of the 39,500-dalton protein released from COMC at each extraction temperature.

In a separate experiment, the insoluble material remaining after treating COMC in SDS buffer at 37 and 60°C for 1 h and at 100°C for 10 min was fixed with glutaraldehyde and processed for electron microscopy.

Purification of the 39,500-dalton outer membrane protein. Isolated COMC prepared from 25 to 30 mg of L2 EB protein were suspended in 5 ml of 2% SDS buffer and incubated at 37°C for 1 h. This suspension was centrifuged at 100,000 × g for 1 h, and the soluble supernatant fraction was collected. This SDS extract, enriched in the 39,500-dalton protein, was then dialyzed against 200 volumes of 0.01 M sodium phosphate, pH 6.4, containing 1 mM dithiothreitol and 0.1% SDS (column equilibration buffer) for 24 h with several changes of dialysate. This extract was fractionated by hydroxylapatite chromatography in the presence of SDS by the technique of Moss and Rosenblum (28). Briefly, the dialyzed extract (8 to 10 ml) was applied to a preequilibrated hydroxylapatite column (0.9 by 30 cm). The column was washed with 100 ml of equilibration buffer and eluted with a 150-ml linear gradient of 0.1 to 0.6 M sodium phosphate, pH 6.4, containing 1 mM dithiothreitol and 0.1% SDS. The column eluate was collected in 40-drop fractions at a flow rate of 5 to 6 ml/h and spectrophotometrically monitored at 280-nm absorbance. Fractions showing positive absorbance were analyzed by SDS-PAGE. Polyacrylamide gels were stained with Coomassie blue for protein and Stains-All to detect nucleic acid and glycolipid moieties. The phosphate molarity of every 10th column fraction was determined by measuring total phosphorus (1) and converting to phosphate molarity by using a standard curve prepared with known sodium phosphate standards. Those fractions that contained only the 39,500-dalton protein were pooled and concentrated to a 1- to 2-ml volume by vacuum dialysis against 0.05 mM Tris-hydrochloride, pH 8.5, containing 0.15 M NaCl and 0.1% SDS. These concentrated preparations were used for performing analytical assays to test for protein purity and as a source of immunogen for the preparation of mouse antisera.

Antisera. Swiss Webster mice strain ICR (Charles River Co., Baltimore, Md.) were immunized subcutaneously on day 0 with 30 μg of purified MP39.5 emulsified with Freund incomplete adjuvant. Immunizations were repeated with the same amount of purified protein administered subcutaneously without adjuvant on days 16 and 27. Mice were bled by cardiac puncture 5 days after each booster immunization (days

21 and 32, respectively). The reactivity and specificity of the pooled sera collected from each bleeding was evaluated by indirect immunofluorescence (micro-IF).

Micro-IF. The micro-IF method of Wang (37) developed for the serological classification of *C. trachomatis* isolates was used to determine the specificity of antiserum raised against purified outer membrane protein. Class-specific antibodies were measured with fluorescein isothiocyanate-conjugated goat antiserum specific against mouse immunoglobulin M (IgM) (μ-chain specific) and IgG (γ-chain specific) (Cappel Laboratories, Cochranville, Pa.). *C. trachomatis* serotypes A, B, Ba, C, D, E, F, G, H, I, J, K, L1, L2, and L3 were tested, as well as the mouse pneumonitis strain. The *C. psittaci* strains tested were: feline pneumonitis, guinea pig inclusion conjunctivitis, and 6BC.

RESULTS

Extrinsic radioiodination of *C. trachomatis* EB. Purified EB (Fig. 1) of the *C. trachomatis* L2, E, and C serotypes were extrinsically radioiodinated by lactoperoxidase-catalyzed iodination. These serotypes were selected for study since they are representative serotypes of the two major *C. trachomatis* serogroups. The L2 and E serotypes belong to the *C. trachomatis* B serological complex, and the C serotype belongs to the C serological complex (9). Whole-cell lysates of these EB were prepared, standardized according to radioactivity, and then subjected to SDS-PAGE. The protein profiles were analyzed by autoradiography and Coomassie brilliant blue staining. Figure 2a shows the results of the autoradiogram. The most intensely radioemitting protein band for each serotype was a 39,500-dalton protein. Other less intensely radioiodinated proteins were observed. These proteins were also shared by each serotype and had apparent subunit molecular weights of 105,000, 68,000, 57,000, 52,000, and 45,000. The 105,000-dalton protein was much less intensely radioiodinated for the E serotype. The Coomassie blue-stained gel protein profiles (Fig. 2b) revealed that the 39,500-dalton surface protein was quantitatively the most abundant protein constituent for each of the three *C. trachomatis* serotypes.

The 39,500-dalton surface protein had the following properties that are characteristic of the major or matrix outer membrane proteins of certain gram-negative organisms: (i) its apparent surface location; (ii) its apparent subunit molecular weight of 39,500 by SDS-PAGE; and (iii) its quantitative dominance in polypeptide profiles of whole-cell lysates. Therefore we have called this protein MP39.5, signifying "major outer membrane protein with an apparent subunit molecular weight of 39,500 daltons."

Effects of various detergents on the extraction of MP39.5 from intact EB. To isolate and purify MP39.5, a common approach would be to first obtain pure chlamydial outer membranes and then extract the protein from these preparations. This approach was not feasible since chlamydiae are insensitive to the action of lysozyme (36) that is routinely used to prepare spheroplasts from which outer membranes are isolated after osmotic lysis. Also, the extremely small quantities of purified *C. trachomatis* organisms that can be practically obtained discouraged investigations directed at isolating outer membranes from mechanically disrupted organisms, since we felt that this method would result in very small yields of homogeneous membrane. Therefore, we investigated the ability of various detergents to extract MP39.5 from intact chlamydiae. The L2 serotype was used for these and all subsequent studies. The ability of nonionic (Triton X-100), dipolar ionic (Zwittergent), mildly anionic (deoxycholate and Sarkosyl), and strongly anionic (SDS) detergents to extract MP39.5 form intact L2 EB was examined by SDS-PAGE of the soluble and insoluble protein fractions obtained after treating EB with each detergent for 1 h at 37°C (Fig. 3). Figure 3a shows the soluble supernatant fractions obtained after treating intact EB with each of the above detergents. The only effective detergent for extracting MP39.5 was SDS (Fig. 3a, lane 6), and, as expected, it also extracted more of the total EB proteins than any of the other detergents studied. Sarkosyl extracted only a small amount of MP39.5 but was nearly as efficient as SDS in extracting the other EB proteins. Zwittergent, deoxycholate, and Triton X-100 also failed to extract significant amounts of MP39.5 and were considerably less effective than Sarkosyl in extracting the additional EB proteins. These data showed that none of the detergents selectively extracted MP39.5 from intact EB, and that distinct variations in the ability of these detergents to extract other EB proteins existed. The insoluble EB fractions obtained from the initial detergent treatments were reextracted in 2% SDS for 1 h at 37°C, and their soluble supernatants were analyzed by SDS-PAGE (Fig. 3b). The Sarkosyl-insoluble pellet was enriched in MP39.5 (Fig. 3b, lane 5), and this protein was quite homogeneous in comparison with the other insoluble EB fractions. Only the SDS-insoluble EB pellet contained less contaminating proteins, but the quantity of MP39.5 which it contained was approximately half that of the Sarkosyl-insoluble fraction. These results showed that sequential anionic detergent treatment of intact L2 EB with Sarkosyl, followed by SDS, was the most desirable procedure for isolating MP39.5. This procedure resulted in a nearly quantitative recovery of the protein from intact EB in a

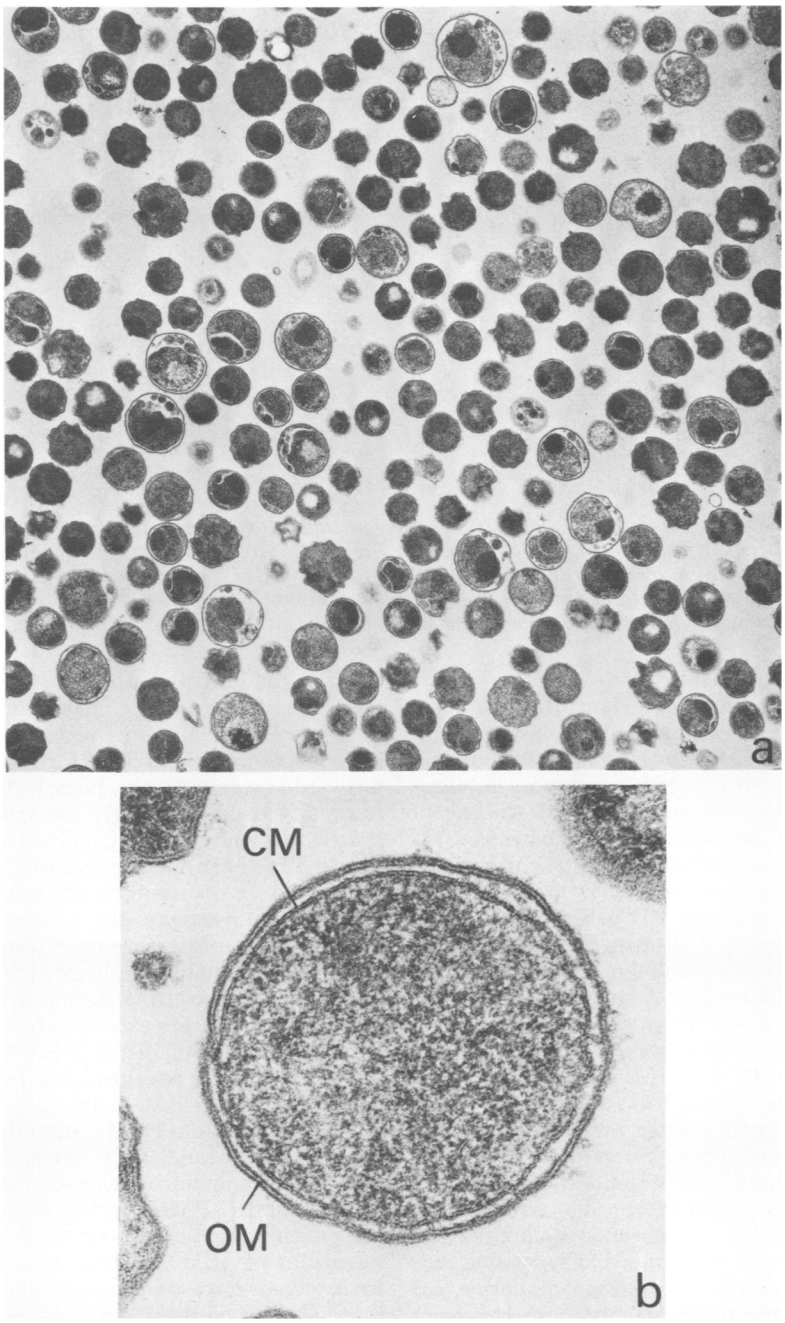

FIG. 1. *Electron microscope photomicrographs of thin sections of purified C. trachomatis serotype L2 EB. (a) Field view (×12,600) that is representative of the purity of C. trachomatis EB used. The preparation consists primarily of electron-dense EB. The larger particles with a dense core and a more diffuse matrix are chlamydial intermediate forms. In our hands these intermediate forms copurified with the EB. (b) Higher magnification (×150,000) of a single EB showing the electron-dense tracked layers of the unit outer membrane (OM) and cytoplasmic membrane (CM) structures. Note the absence of any structural evidence of a peptidoglycan layer between the OM and CM.*

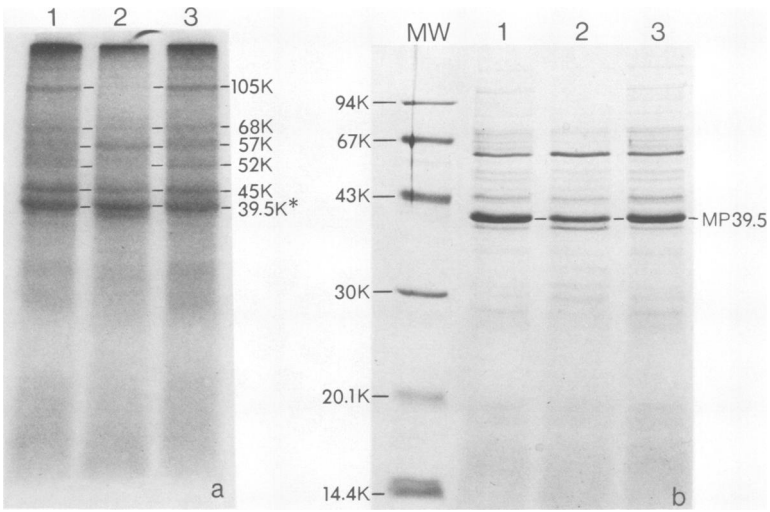

FIG. 2. *SDS-PAGE protein profiles of extrinsically radioiodinated elementary bodies of C. trachomatis serotypes L2, E, and C. The protein profiles shown are EB whole-cell lysates prepared by SDS solubilization of intact organisms. (a) Autoradiogram after a 24-h exposure. Lane 1, L2 serotype; lane 2, E serotype; lane 3, C serotype. (b) The same gel stained with Coomassie brilliant blue. Lanes correspond to those in panel a. The left lane contains molecular weight markers. The MP39.5 band is illustrated.*

relatively homogeneous form.

Since Sarkosyl has been shown to selectively solubilize the cytoplasm and cytoplasmic membrane proteins of gram-negative bacteria (6), and because Sarkosyl effected extensive solubilization of proteins from intact EB with the exception of MP39.5, we were interested in what effect this detergent had on the ultrastructure of the chlamydiae. To determine this, thin sections of the Sarkosyl-insoluble EB pellets were examined by transmission electron microscopy. This material (Fig. 4a) consisted of uniform particles with a single intact double-tract unit membrane of a size and morphology characteristic of the native EB outer membrane (see Fig. 1b). The strict association of the MP39.5 with this Sarkosyl-insoluble material proves that the membrane is chlamydial outer membrane and not cytoplasmic membrane. No cytoplasm or cytoplasmic membrane was visualized, nor could an electron-dense peptidoglycan-like structure be identified (Fig. 4b). The absence of a peptidoglycan-like structure was somewhat surprising considering the excellent retention of native EB morphology by these membranes. However, considering the fact that other investigators have failed to demonstrate *N*-acetylmuramic acid in highly purified EB preparations (8, 23), suggesting the absence of a typical bacterial murein sacculus, it seemed possible to us that some other macromolecular structure might be responsible for providing this observed membrane structure and particle rigidity. Therefore, we named the material in the Sarkosyl-insoluble fraction the chlamydial outer membrane complex to indicate that it consisted of an outer membrane associated with some type of rigidity-conferring structure, which for the chlamydiae has yet to be definitively characterized. Because MP39.5 was quantitatively the most abundant protein present in COMC (Fig. 3b, lane 5), we were interested in determing the role this protein might play in maintaining the structure of the COMC. To investigate this, we examined both the effect of temperature on extracting MP39.5 and the accompanying ultrastructural changes in COMC that occurred during this treatment. Isolated COMC were extracted for 1 h at 37, 45, 60, and 80°C and for 10 min at 100°C in 2% SDS, and their soluble and insoluble fractions were analyzed by SDS-PAGE. In a separate experiment, the insoluble COMC material remaining after the 37, 60, and 100°C extraction temperature was examined ultrastructurally. Figure 5 shows the SDS-PAGE protein profiles obtained after each extraction temperature. The relative quantities of MP39.5 released from COMC at each temperature were determined by densitometric scans of the stained electropherogram (data not shown). Analysis of the 37°C soluble and insoluble fractions showed that approximately 80% of MP39.5 was extracted from COMC at 37°C for 1h (Fig. 5, lanes 1 and 1a). Increasing the extraction temperature to 45°C (Fig. 5, lanes 2 and 2a) did not significantly change the results; however, after 1 h at 60°C (Fig. 5, lanes 3 and 3a) or 80°C (Fig. 5, lanes 4 and 4b), or after 10 min at 100°C (Fig. 5, lanes

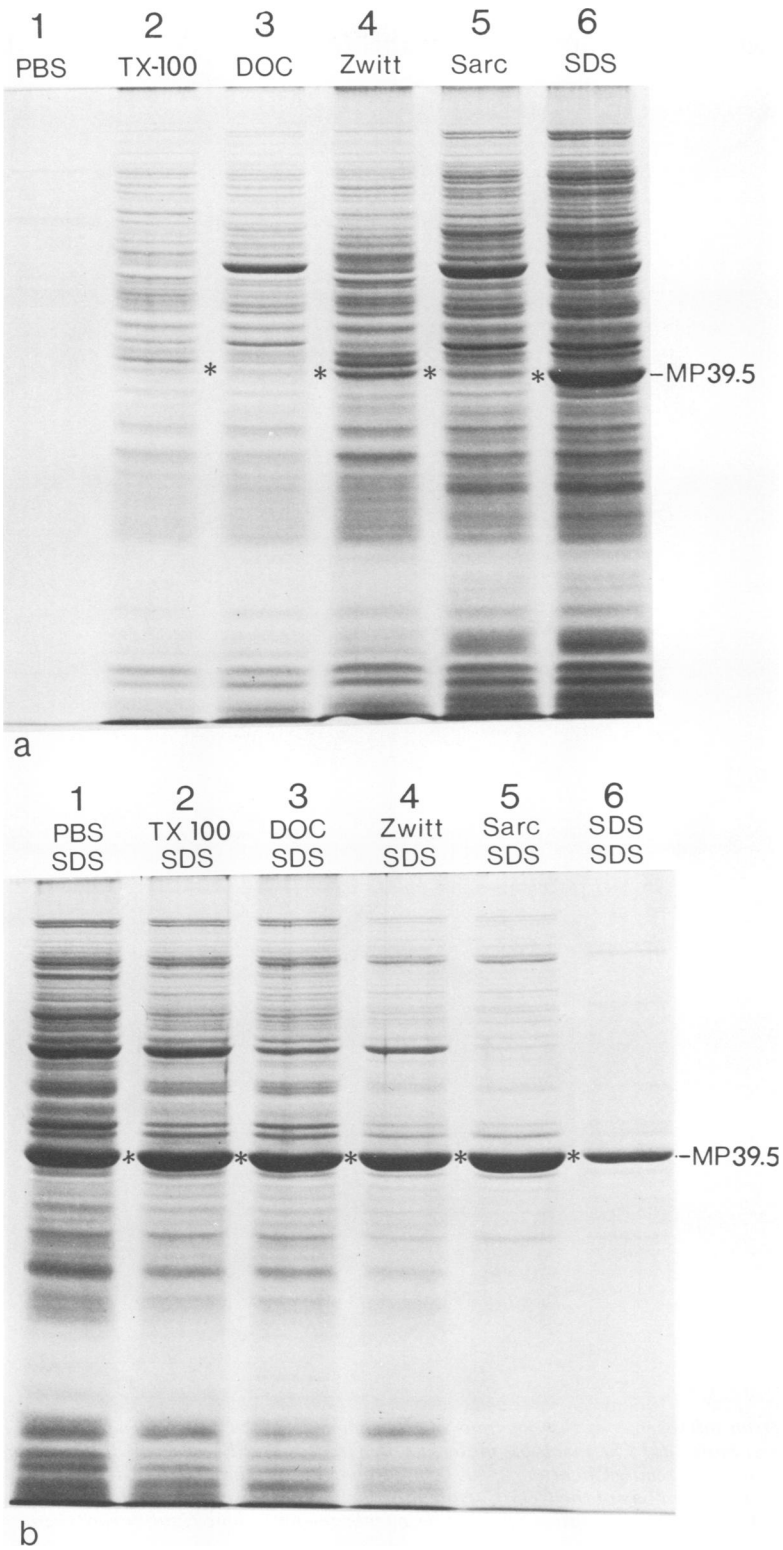

FIG. 3. *SDS-PAGE protein profiles demonstrating the effect of different detergents on extracting MP39.5 from C. trachomatis serotype L2 EB. (a) SDS-PAGE patterns of the soluble supernatants recovered after each detergent treatment. Lane 1, PBS-EDTA buffer control; lane 2, 2% Triton X-100; lane 3, 2% deoxycholate; lane 4, 2% Zwittergent; lane 5, 2% Sarkosyl; lane 6, 2% SDS. (b) SDS-PAGE protein patterns of the insoluble EB fractions. The insoluble EB pellets were treated with 2% SDS for 1 h at 37°C. The soluble supernatant fractions obtained after centrifugation were analyzed by SDS-PAGE. Lanes 1 through 6 correspond to those in panel a.*

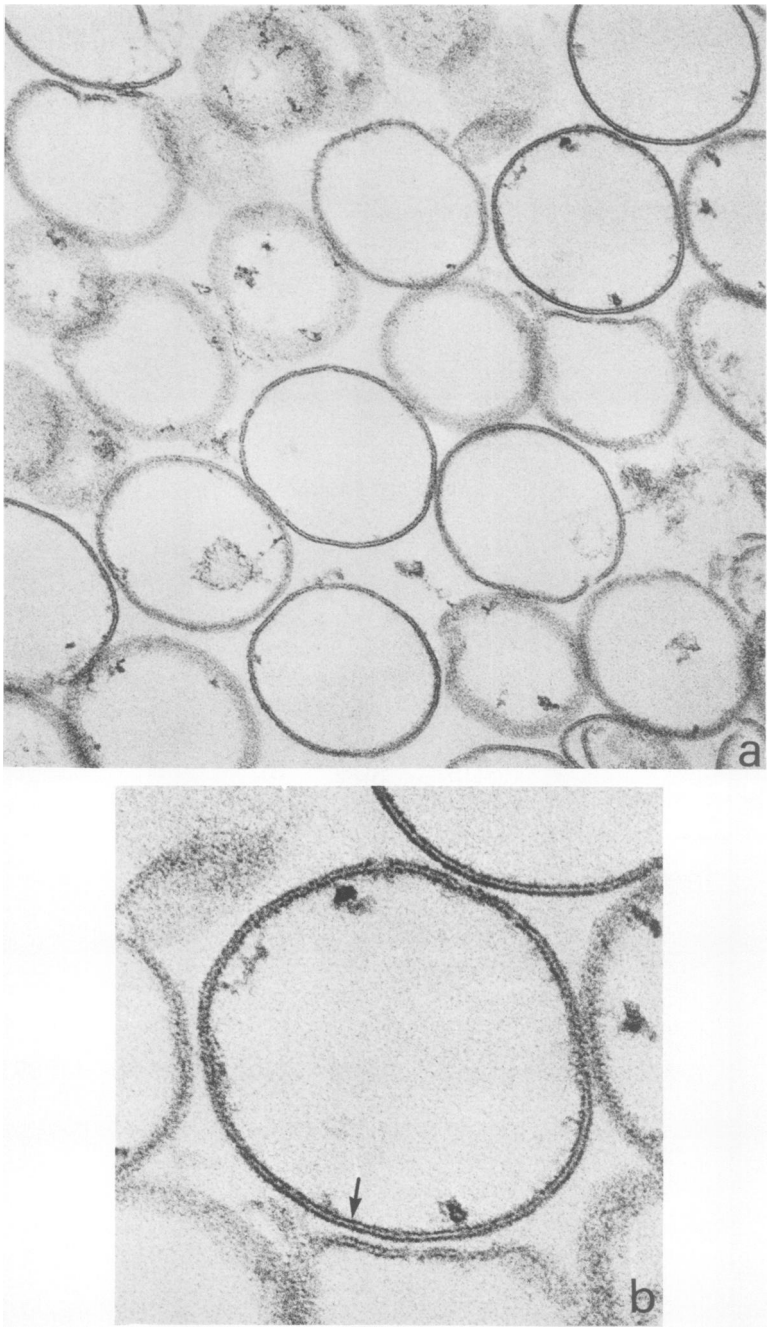

FIG. 4. *Electron microscopy of thin sections of C. trachomatis L2 COMC prepared by treating intact EB with 2% Sarkosyl buffer. (a) The insoluble material after treating L2 EB with 2% Sarkosyl buffer at 37°C for 1 h (×72,000). (b) Higher magnification (×105,000) demonstrating the electron-dense unit outer membrane (arrow) and rigid morphology of the COMC. Note that the morphology of the COMC is consistent with intact EB (Fig. 1b), indicating the association of the outer membrane with some rigidity-conferring macromolecular structure.*

5 and 5a), virtually all (99.6%) of MP39.5 was found in the soluble supernatant fractions. A densitometric scan of the 100°C soluble fraction showed that 61% of the total COMC-associated protein was MP39.5. Because at least a portion of MP39.5 was radioiodinated by lactoperoxi-

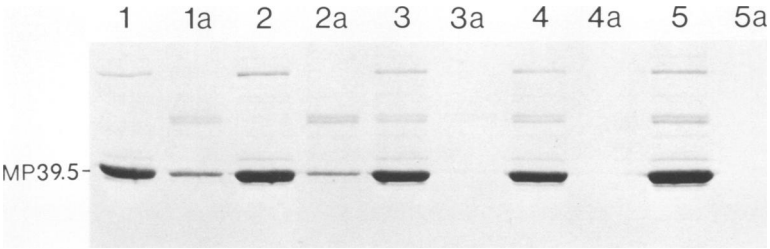

FIG. 5. *Effect of temperature on SDS extraction of MP39.5 from COMC. Equal concentrations of COMC were extracted with equal volumes of 2% SDS buffer under one of the following conditions: 37, 45, 60, or 80°C for 1 h or 100°C for 10 min. Each suspension was centrifuged at 100,000 × g for 1 h at 37°C. The insoluble COMC pellets from each temperature treatment were resuspended in 2% SDS buffer and incubated at 100°C for 10 min. The protein profiles of the soluble and insoluble fractions were compared by SDS-PAGE. Lane 1, 37°C soluble supernatant (S); lane 1a, 37°C insoluble pellet (P) boiled in SDS buffer; lane 2, 45°C (S); lane 2a, 45°C (P); lane 3, 60°C (S); lane 3a, 60°C (P); lane 4, 80°C (S); lane 4a, 80°C (P); lane 5, 100°C (S); lane 5a, 100°C (P). A densitometric scan of the 100°C soluble protein profile (lane 5) showed that MP39.5 accounted for 61% of the total COMC protein. Extraction at 37°C for 1 h solubilized 80% of the total MP39.5 (lanes 1 and 1a). Complete extraction of MP39.5 was achieved only at 60°C or higher. Only the upper half of the gel is shown.*

dase-catalyzed iodination, and the protein was found to be strongly associated with COMC as shown by its disassociation properties in the presence of SDS, we believe that it may be a transmembrane protein. The MP39.5 band did not vary in its electrophoretic migration characteristics with different conditions of heating during solubilization of the COMC in SDS. This was true even when solubilization was carried out at 100°C for 30 min. Three properties of MP39.5 closely resemble those of certain matrix proteins of gram-negative bacteria: (i) its quantitative dominance in the EB outer membrane; (ii) its apparent transmembrane character; and (iii) its inability to be heat modified.

The extraction of MP39.5 was found to correlate with changes in the COMC ultrastructure. At 37°C, a temperature at which 80% of MP39.5 was solubilized, the number of intact COMC was greatly diminished, and primarily disrupted COMC or their membrane fragments were observed in the insoluble pellet (Fig. 6). Accompanying this change in COMC ultrastructure were aggregates of amorphous material that were not observed in thin sections of COMC before SDS treatment. The nature of this insoluble material is unclear. It is doubtful that it is nucleic acid since nucleases were used in the isolation of COMC. At 60°C, a temperature at which all of MP39.5 was solubilized from COMC, the insoluble COMC pellet contained only the amorphous aggregates seen in Fig. 6. No structures resembling intact COMC or their membrane fragments were observed. These findings suggest that MP39.5 plays an important role in maintaining both the membrane integrity and structural morphology of the COMC. The amorphous material present in thin sections of insoluble COMC pellets extracted at 37 and 60°C was also found in the insoluble COMC material that was heated at 100°C. The fact that this material remained insoluble despite treatment with SDS at 100°C and that no additional proteins were detected by SDS-PAGE after this treatment (Fig. 5, lanes 5 and 5a) suggest that it is not protein bound together by strong hydrophobic or ionic interactions. If the material is protein, it must be associated through covalent bonding. Since the appearance of this amorphous material correlated with the extraction of MP39.5 and also accompanied the subsequent ultrastructural changes in the COMC, its role alone, or in combination with MP39.5, cannot be excluded as a possible explanation for maintaining the structural morphology of the COMC. These results suggest that the structure of the COMC of *C. trachomatis* organisms is not dependent on a bacterial mucopeptide-like sacculus. The structural integrity of the bacterial mucopeptide is unaffected by the conditions used above to disassociate the COMC, and in fact similar conditions are used for isolating bacterial murein sacculi (2).

Isolation and purification of MP39.5. We took advantage of the sequential anionic detergent treatment described above as a preparatory step to isolate large quantities of relatively homogeneous MP39.5 from COMC. Approximately 25 to 30 mg of protein of whole L2 EB was extracted with Sarkosyl to prepare COMC. For these experiments, nucleases were not used during the isolation of COMC.

MP39.5 was extracted from COMC with 2% SDS buffer for 1 h at 37°C. After centrifugation, the soluble fraction (enriched in MP39.5) was dialyzed and loaded onto a preequilibrated hydroxylapatite column. We chose the 37°C extraction temperature for the following reasons:

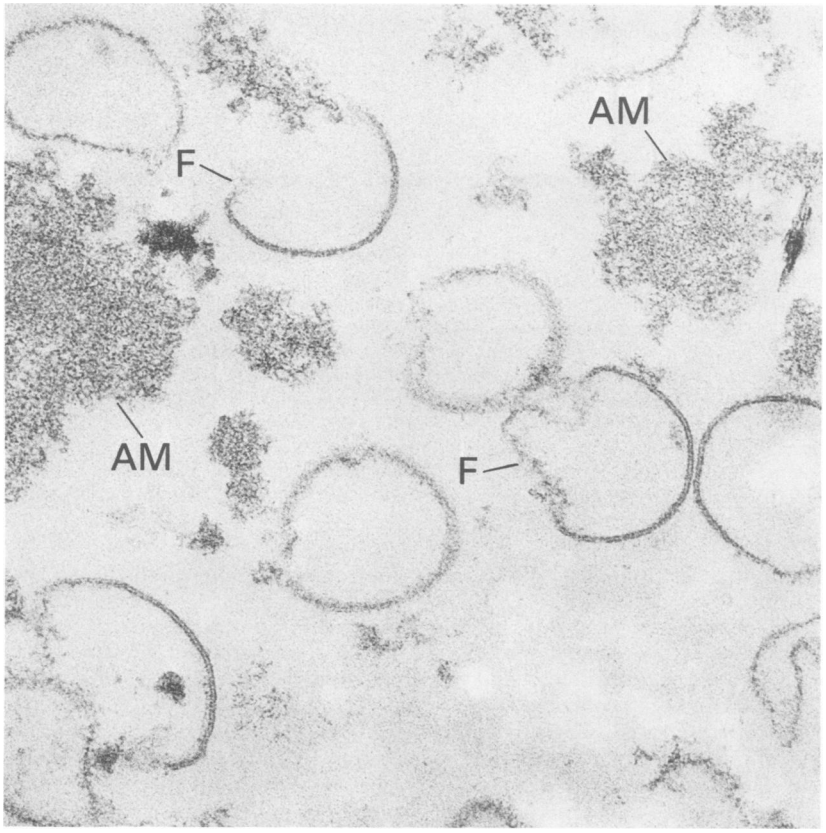

FIG. 6. *COMC after treatment with 2% SDS buffer at 37°C for 1 h. Note that the integrity of the outer membranes shown in Fig. 4b has been significantly diminished. In addition, the COMC are mostly fragmented (f) and have lost their characteristic morphology. This change in ultrastructure was accompanied by the solubilization of large quantities of MP39.5 from the COMC (Fig. 5, lane 1). In addition to the structural changes in COMC after SDS treatment, the insoluble pellet also contained the amorphous material (AM) illustrated in the figure.*

(i) under these conditions the majority (80%) of MP39.5 was extracted from COMC, and (ii) since we were interested in the antigenic properties of the protein, we felt that this temperature would reduce the possibility of extensive denaturation that may have occurred in the presence of SDS at the more efficient extraction temperatures (i.e., 60°C and higher). The column was washed with 100 ml of equilibration buffer and then eluted with a 0.1 to 0.6 M linear phosphate gradient. Column fractions were monitored at 280-nm absorbance. Three peaks were eluted from the column (Fig. 7). Peak I eluted at a concentration of approximately 0.18 M phosphate. The fractions of peak I contained no detectable protein as determined by SDS-PAGE. Spectrophotometric analysis ($OD_{280/260}$) of the pooled fractions of this peak indicated that this material was chlamydial nucleic acid. Peak II was eluted at approximately 0.3 M phosphate. SDS-PAGE of the eluted fractions from peak II showed these fractions to contain only MP39.5 (Fig. 8, fractions 55 through 65), with the majority of MP39.5 eluting in fractions 58 through 61. No other proteins could be detected in these fractions. The material appeared to be free of glycolipid and nucleic acid, since staining the gel with Stains-All failed to detect these moieties. After the elution of MP39.5, the gradient was discontinued and 0.6 M phosphate buffer was added to the column. By using this procedure, the remaining chlamydial proteins bound to the column were eluted as a single peak (Fig. 7, peak III). This peak contained a heterogeneous mixture of high-molecular-weight proteins (Fig. 8, fractions 105 through 109) plus a small amount of MP39.5. The proteins of peak III could be eluted from the column by continuing the linear phosphate gradient; however, by doing this they were distributed over a large fractionation volume and were therefore not readily detected after SDS-PAGE.

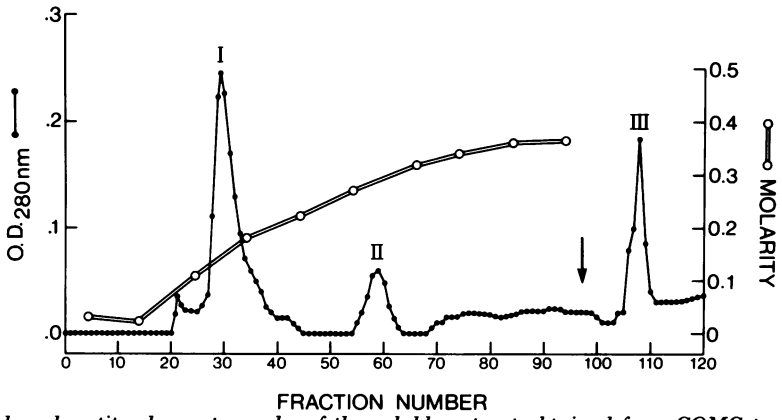

FIG. 7. *Hydroxylapatite chromatography of the soluble extract obtained from COMC treated with SDS buffer. An SDS extract containing approximately 4 mg of protein was dialyzed against equilibration buffer and adsorbed to a preequilibrated hydroxylapatite column (0.9 by 30 cm). After an equilibration buffer wash to remove unbound material, a linear phosphate gradient was applied (0.1 to 0.6 M). Fractions were monitored spectrophotometrically at 280 nm and by SDS-PAGE. The arrow shows where the gradient was stopped and 0.6 M phosphate buffer was added to elute all remaining column-bound chlamydial proteins.*

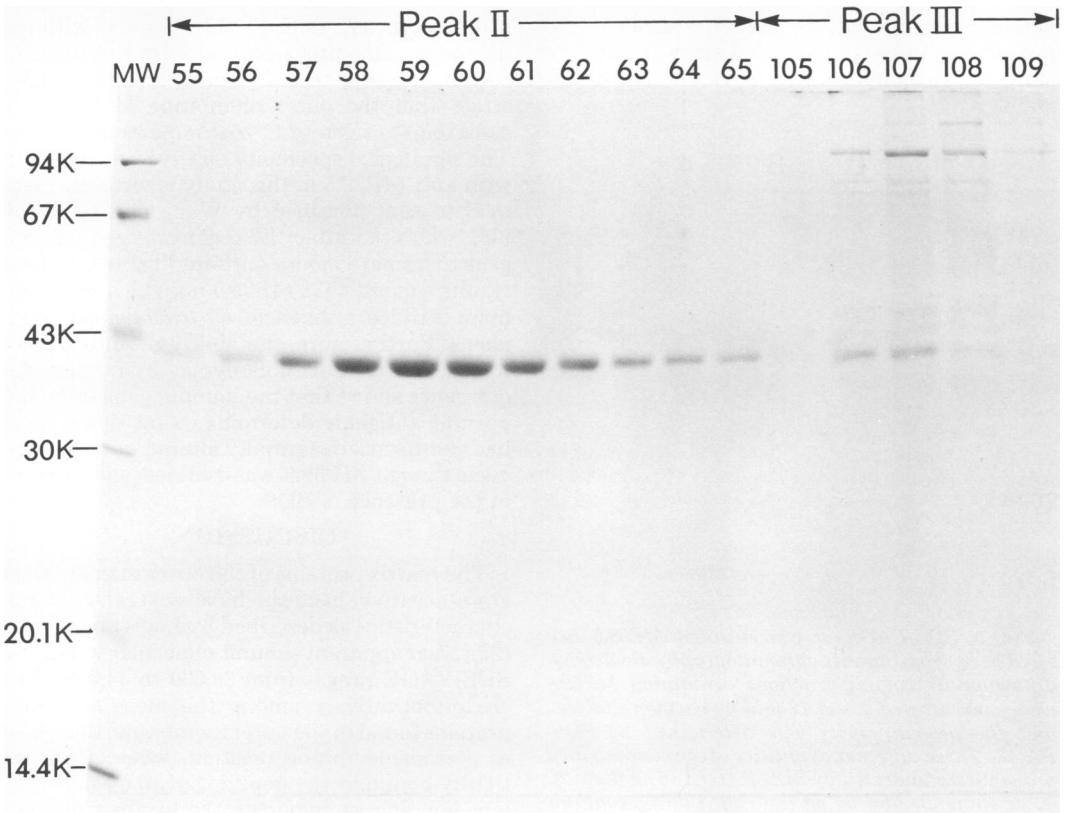

FIG. 8. *SDS-PAGE of fractions eluted from hydroxylapatite chromatography. Fractions corresponding to peaks II and III of Fig. 5 are shown. No protein was detected by SDS-PAGE of fractions contained in peak I. Spectrophotometric analysis of peak I ($OD_{280/260}$) revealed it to be composed of chlamydial nucleic acid. Peak II (fractions 55 through 65) contained only MP39.5. No contaminating proteins were visualized in these fractions. Neither glycolipid or nucleic acid could be detected in these fractions by staining the gel with Stains-All. Peak III (fractions 105 through 109) that was eluted after the addition of 0.6 M phosphate contained high-molecular-weight chlamydial proteins and a small amount of MP39.5.*

Fractions containing MP39.5 were pooled and concentrated to a 1- to 2-ml volume by vacuum dialysis. Approximately 1.4 mg of protein was recovered after concentration. Although this quantity of purified MP39.5 is small compared with the recovery of outer membrane proteins from readily cultivatable organisms, we consider these yields exceptional considering that only 25 to 30 mg of EB protein was used as starting material.

Figure 9 shows the protein purity of the pooled column fractions containing MP39.5 after concentration. Some contaminating proteins were observed after concentration. The most prominent contaminant was a 36,000-dalton protein that apparently copurified with MP39.5. Densitometric scans of the stained gel showed that MP39.5 accounted for 91% of the total protein. The 36,000-dalton protein accounted for 3% of the total protein (data not shown). No 2-keto-3-deoxyoctanoic acid was detected in the concentrated preparation, indicating the absence of chlamydial lipopolysaccharide. MP39.5 appeared not to be glycosylated since it failed to stain for carbohydrate by periodic acid-Schiff stain.

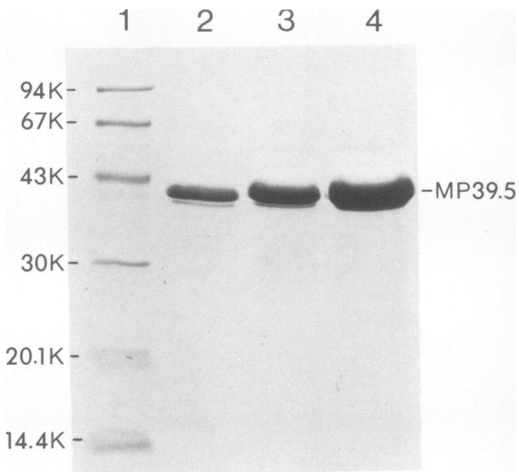

FIG. 9. *SDS-PAGE of concentrated MP39.5 isolated by hydroxylapatite chromatography. Pooled hydroxylapatite column fractions containing MP39.5 were concentrated 10- to 12-fold by vacuum dialysis, and the protein purity was determined by SDS-PAGE. Three different quantities of the concentrated protein were analyzed by SDS-PAGE: 16 μg (lane 2); 32 μg (lane 3); and 64 μg (lane 4). Lane 1 contains molecular weight markers. A densitometric scan of the stained gel (lane 3) showed that 91% of the total protein in the concentrated preparation was MP39.5. The most prominent contaminant was a 36,000-dalton protein that migrated immediately below the MP39.5 band. This protein comprised less than 3% of the total protein profile.*

Serological specificity of anti-MP39.5 sera. The micro-IF procedure that is used for the serological classification of *C. trachomatis* isolates was used to examine the specificity of mouse anti-MP39.5 sera. Table 1 shows the antibody responses after the second (day 21) and third (day 32) booster immunizations with 30 μg of purified MP39.5. The titers shown represent IgG antibody only; no fluorescence was detected with anti-IgM-specific conjugate. The antibody response after the second immunization was found to be primarily homotypic in that significant titers were present only against the L2 serotype. However, a distinct antibody response was observed with the Ba, D, E, K, L1, and L3 serotypes at lower antiserum dilutions. With repeated immunization, both the homologous and cross-reacting antibody titers increased substantially; however, the same serotype specificity that was initially observed was maintained throughout the immunization period. Antiserum against MP39.5 did not react with *C. trachomatis* serotypes A, B, C, F, G, H, I, and J, with the mouse pneumonitis strain, or with the three *C. psittaci* strains tested. These results demonstrate that the outer membrane MP39.5 is a serogroup antigen of *C. trachomatis* organisms. The serological specificity observed by micro-IF with anti-MP39.5 in this study is virtually identical to that obtained by Wang and Grayston (38), who used intact L2 organisms as immunogens to prepare mouse antisera (Table 1). These results suggest that MP39.5 may be a serodominant surface antigen of *C. trachomatis* organisms. Furthermore, the ability of anti-MP39.5 to react with intact chlamydiae by immunofluorescence shows that the immunogenicity of important antigenic determinants on this protein had not been substantially altered or denatured even though MP39.5 was isolated and purified in the presence of SDS.

DISCUSSION

The matrix proteins of the outer membrane of gram-negative bacteria have several common characteristics as described by Lugtenberg et al. (22): their apparent subunit molecular weight by SDS-PAGE ranges from 33,000 to 44,000; they are quantitatively among the most abundant proteins found in the outer membrane; they exist in a transmembrane position, where they are tightly bound to the peptidoglycan by noncovalent bonding as demonstrated by their disassociation in the presence of SDS; and their electrophoretic mobility by SDS-PAGE is not affected by heating. The *C. trachomatis* protein we have designated MP39.5 closely resembles these matrix proteins.

One of the functions attributed to matrix pro-

TABLE 1. *Serological cross-reactivity of mouse antisera to L2 MP39.5 and L2 EB with other C. trachomatis serotypes or C. psittaci strains as determined by micro-IF*

Organism	Serotype or strain	Antiserum against: L2 MP39.5[a] Titer after 2nd booster immunization (day 21)	L2 MP39.5[a] Titer after 3rd booster immunization (day 32)	L2 EB[b]
C. trachomatis	A	—[c]	—	—
	B	—	—	—
	Ba	8 (13)[d]	128 (25)	13
	C	—	—	—
	D	8 (13)	128 (25)	6–13
	E	8 (13)	64 (13)	6–13
	F	—	—	—
	G	—	—	ND[e]
	H	—	—	ND
	I	—	—	ND
	J	—	—	ND
	K	8 (13)	128 (25)	ND
	L1	8 (13)	128 (25)	13
	L2	64 (100)	512 (100)	100
	L3	8 (13)	128 (25)	13–25
	Mouse pneumonitis	—	—	ND
C. psittaci	6B	—	—	ND
	Feline pneumonitis	—	—	ND
	Guinea pig inclusion conjunctivitis	—	—	ND

[a] Reciprocal of the highest dilution of antiserum (starting at 1:8) showing fluorescence. Antibody titers are IgG only; no fluorescence was observed with anti-IgM-specific conjugate.

[b] From published data of Wang and Grayston (38). Cross-reactions are expressed as percentages in relationship to the homologous titer.

[c] —, No fluorescence detected at a 1:8 dilution.

[d] Percentage of the homologous titer is expressed in parentheses. Titers were converted to a percentage so that comparisons could be made with the serological cross-reactivity of mouse antiserum raised against intact L2 EB in reference 38.

[e] ND, Not done; serotypes G, H, I, J, and K had not yet been identified.

teins is that they are transmembrane diffusion pores or "porins" (31, 32). They also may contribute to the structural integrity of the gram-negative bacterial cell because of their strong interaction with the peptidoglycan (4). For example, the matrix protein 1a of *Escherichia coli* B remains associated with its peptidoglycan in the presence of 2% SDS at 60°C (35). Similar proteins of *Pseudomonas aeruginosa* are completely extracted at 55°C but remain associated with the peptidoglycan at <50°C (27). MP39.5 is disassociated from COMC in 2% SDS at 37°C (80% complete extraction), and thus this protein interacts with the COMC with a much lower affinity than the matrix proteins do with their peptidoglycan. When the matrix proteins are extracted from gram-negative bacteria, the murein sacculus maintains the organism's basic morphology. In contrast, the extraction of MP39.5 from COMC results in a complete loss of structural integrity.

This difference most likely reflects the lack of a peptidoglycan in chlamydiae as demonstrated by their insensitivity to lysozyme (36) and the failure to detect *N*-acetylmuramic acid or quantities of other amino sugars in purified EB that would reflect structural significance (8, 23). However, the rigid morphology that is characteristic of the chlamydial EB and the sensitivity of chlamydiae to penicillin (18, 25, 39) and D-cycloserine (30) implies that a tetrapeptide structure similar to that of the bacterial cell wall is present. It has been suggested by Garrett et al. (8) that the chlamydiae have a unique skeletal structure and that the chlamydial tetrapeptide may be covalently bound to some other macromolecule other than polysaccharide. It is possible that the amphorous material we observed after disassociation of MP39.5 from COMC may be this structure that has collapsed upon itself after removal of MP39.5. The biochemical nature of this material is unclear. It is interesting to speculate, however, that it may be a covalently bound protein and that the association of MP39.5 with this protein skeleton through strong hydrophobic and ionic interac-

tions provides the rigidity and structural integrity of the chlamydial cell. Such a unique protein-protein (rather than protein-polysaccharide) association may ultimately serve to differentiate chlamydiae from other bacteria just as their growth cycle places them apart from all other microorganisms. The uniqueness of such a structure may be functionally related to the uniqueness of the chlamydial growth cycle. Early in the cycle the chlamydial EB reorganizes into a metabolically active reticulate particle. During this reorganizational period the reticulate particle increases in size, to approximately five times that of the EB. The outer membrane not only enlarges during this process, but it also must change its permeability properties to allow for the uptake of large exogenous metabolites such as nucleoside triphosphates (10, 40) that the intracellular reticulate particle is unable to synthesize (29). This demonstrates that the chlamydial outer membrane and its associated skeletal structure have unusual physiological and structural properties. The type of outer membrane skeletal structure which we hypothesize may provide for greater functional flexibility than would be expected if the chlamydiae possessed a rigid peptidoglycan. Conservation of such a simple and easily modified structure would be consistent with the genetic composition of *Chlamydia* since their genome is only half that of rickettsia and one-eighth that of *E. coli* (17).

It is likely that previous publications referring to the isolation of chlamydial cell walls by using anionic detergents (12, 23) may have been based upon incomplete extraction of outer membrane proteins. Thus the "cell walls" described by these investigators may have been analogous to the COMC that we identified in this study.

The isolated MP39.5 appears to maintain at least some of its native antigenic properties even though the protein was purified in the presence of SDS. When antiserum against MP39.5 was assayed by the micro-IF test (the standard procedure for serological classification of *C. trachomatis*), we found that the serological cross-reactivity was typical of the micro-IF response described by Wang and Grayston (38), who used antiserum prepared against intact L2 organisms. They too found cross-reactions with Ba, E, D, L1, L2, and L3 serotypes. Failure of this antiserum to react with the strains that are present within the C complex suggests that MP39.5 has an antigen common to all members of the B complex and is one of the primary antigens recognized after immunization with viable or whole chlamydial EB. Since SDS did not significantly alter the immunogenic properties of this protein, it seems likely that the antigen(s) being detected is most likely a surface-located sequential determinant of the polypeptide. There is an obvious caveat to the interpretation of the serological results because there were small amounts of contaminating protein present in the concentrated MP39.5 preparation used for immunization (Fig. 9). We think it unlikely that the contaminants could have been responsible for the serological results since the major contaminant, a 36,000-dalton protein, represented less than 3% of the total preparation and the entire immunization course would have introduced less than 3 μg of the contaminating protein into the immunized animals. However, although we think it unlikely that any contaminants were responsible for the observed antibody reactions, we cannot completely eliminate the possibility that this may have contributed to the micro-IF results.

Although it is clear from the above discussion that MP39.5 is a surface-exposed antigen, its role in maintaining the structural integrity of the COMC suggests that it may be an intrinsic protein that spans the chlamydial outer membrane. It is likely that this protein would have other antigenic sites that would not be detected in our assay system, which used intact EB and thus would only measure responses to surface-exposed antigens. It is conceivable that MP39.5 is composed of both hydrophobic and hydrophilic polypeptides. The hydrophilic segment of the protein would be exposed to the aqueous environment, whereas the hydrophobic segment would be embedded in the membrane bilayer. In theory, it does not seem unreasonable to postulate that these two portions of the protein may differ antigenically since their different amphipathic properties could reflect differences in their composition. It would not be surprising to find more common or extensive cross-reaction of the hydrophobic segment among different serotypes or even different chlamydial species, since this portion of the protein would not be exposed to selective environmental pressures such as antibody. In contrast, the exposed hydrophilic segment may modulate its compositon and hence its antigenic character because of selective environmental conditions. This point can only be investigated by a more thorough immunological and biochemical comparison of isolated major outer membrane proteins from different chlamydial serotypes or species.

The *C. trachomatis* L2 strain major outer membrane protein can be easily isolated and purified by a simple sequential procedure involving anionic detergent treatment of purified intact organisms followed by hydroxylapatite chromatography. It is present in relatively large quantities representing approximately 60% of the COMC and permitting yields of 1 to 2 mg of almost homogeneous material from purified

whole organisms containing 25 to 30 mg of protein. Because it appears to be a shared antigen common to a major serotype complex of *C. trachomatis* organisms, it seems to be a reasonable candidate for an antigen to be detected in clinical specimens. Thus, we think MP39.5 and likely its related proteins in other chlamydial strains may be of considerable practical importance if they lend themselves to detection in immunodiagnostic tests and are of considerable basic interest because of their importance in maintaining the structural integrity of the chlamydial EB.

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